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Glucose-induced and nitrogen-starvation-induced peroxisome degradation are distinct processes in *Hansenula polymorpha* that involve both common and unique genes

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Abstract

In the methylotrophic yeast *Hansenula polymorpha* non-selective autophagy, induced by nitrogen starvation, results in the turnover of cytoplasmic components, including peroxisomes. We show that the uptake of these components occurs by invagination of the vacuolar membrane without their prior sequestration and thus differs from the mechanism described for bakers yeast. A selective mode of autophagy in *H. polymorpha*, namely glucose-induced peroxisome degradation, involves sequestration of individual peroxisomes tagged for degradation by membrane layers that subsequently fuse with the vacuole where the organelle is digested. *H. polymorpha pdd* mutants are blocked in selective peroxisome degradation. We observed that *pdd1-201* is also impaired in non-selective autophagy, whereas this process still normally functions in *pdd2-4*. These findings suggest that mechanistically distinct processes as selective and non-selective autophagy involve common but also unique genes.

Keywords: Autophagy; Peroxisome degradation; Methylotrophic yeast; Peroxisome

1. Introduction

Adaptation of cells to changing environments is paralleled by major alterations in the expression of individual genes and may be associated with selective degradation of superfluous proteins. In eukaryotes, selective degradation of individual cytosolic proteins predominantly occurs through the action of the ubiquitin/proteasome system [1]. In contrast, bulk protein and organelle degradation takes place in the lysosome/vacuole via a process known as autophagy [2].

Autophagy has extensively been studied in higher eukaryotes in which two morphologically distinct modes, macro- and microautophagy, have been defined [2]. In macroautophagy portions of the cytoplasm are sequestered from the cytosol by membrane structures, which subsequently fuse with the lysosome resulting in digestion of their contents. During microautophagy cytoplasmic compounds are directly incorporated into the lytic compartment via invaginations of lysosomal membranes.

Autophagic processes also occur in lower eukaryotes like fungi and yeasts. A striking example is the rapid and selective degradation of peroxisomes in methylotrophic yeast [3–5]. In these yeasts peroxisomes that contain key enzymes of methanol metabolism massively develop during growth of cells on methanol. Upon transfer of these cells to glucose- or ethanol-containing media peroxisomes become redundant for growth and are selectively degraded. Similarly, peroxisomes containing enzymes of the β-oxidation pathway are degraded upon a shift of oleic acid-grown *Saccharomyces cerevisiae* cells to glucose media [6,7]. Non-selective autophagy has also been described in yeasts and occurs for instance in *S. cerevisiae* during nutrient limitation [8].

Detailed morphological studies in *Hansenula polymorpha* revealed that upon exposure of methanol-grown cells

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to glucose-excess conditions individual peroxisomes are sequestered from the cytosol by membrane layers that are most likely derived from the endoplasmic reticulum. Subsequently the outer membrane layer fuses with the vacuolar membrane, which results in the uptake and subsequent degradation of the sequestered organelles by vacuolar hydrolases [9]. This process, which resembles macroautophagy in higher eukaryotes, has also been observed in *Pichia pastoris* upon a shift of cells from methanol- to ethanol-containing media [10]. However, when glucose is added to methanol-grown *P. pastoris* cells, peroxisomes are degraded by another mechanism which is similar to microautophagy [10,11]. During this process protrusions that extend from the vacuolar membrane surround and engulf clusters of peroxisomes. Subsequent homotypic fusion of the vacuolar membrane causes the peroxisomes to become enclosed in this organelle.

To understand the molecular mechanisms involved in autophagy, yeast mutants defective in this process have been isolated. *S. cerevisiae* mutants have been described that are defective in non-selective autophagy during nitrogen (N)-starvation (*apg* mutants [12], *aut* mutants [13]). *H. polymorpha* mutants have been isolated that are affected in selective peroxisome degradation (*pdd* mutants [14]). All *H. polymorpha pdd* mutants isolated so far are defective in both glucose- and ethanol-induced peroxisome degradation, but differ in the stage in which the degradation process is affected. *P. pastoris* mutants have been described that are blocked at various stages in glucose-induced peroxisome degradation (*gsa* mutants [10]; *pag* mutants [11]). Some of these mutants are also defective in ethanol-induced peroxisome degradation, which suggests that a partial convergence exists between both morphologically distinct processes in *P. pastoris*.

In this paper we describe non-selective autophagy in the methylotrophic yeast *H. polymorpha*, which is induced by shifting cells from N-excess to N-starvation conditions. Detailed morphological studies revealed that, in contrast to mammalian cells and *S. cerevisiae*, *H. polymorpha* exhibits non-selective autophagy by a process that resembles microautophagy. Moreover, we show that the *H. polymorpha pdd1-201* mutant, which is defective in glucose- and ethanol-induced selective peroxisome degradation, is also blocked in N-starvation-induced non-selective autophagy. In contrast, the *H. polymorpha pdd2-4* mutant is still capable to degrade peroxisomes under such conditions. These findings indicate that the molecular mechanisms involved in selective peroxisome degradation in *H. polymorpha* partially overlap with those of non-selective autophagy, but also involve unique genes.

2. Materials and methods

2.1. Micro-organisms and growth

H. polymorpha wild-type (WT) (NCYC495) and the peroxisome degradation deficient strains *pdd1-201*(*leu1.1*) and *pdd2-4*(*leu1.1*) [14] were used. Cells were grown at 37°C in batch cultures on mineral medium (MM) as described [15]

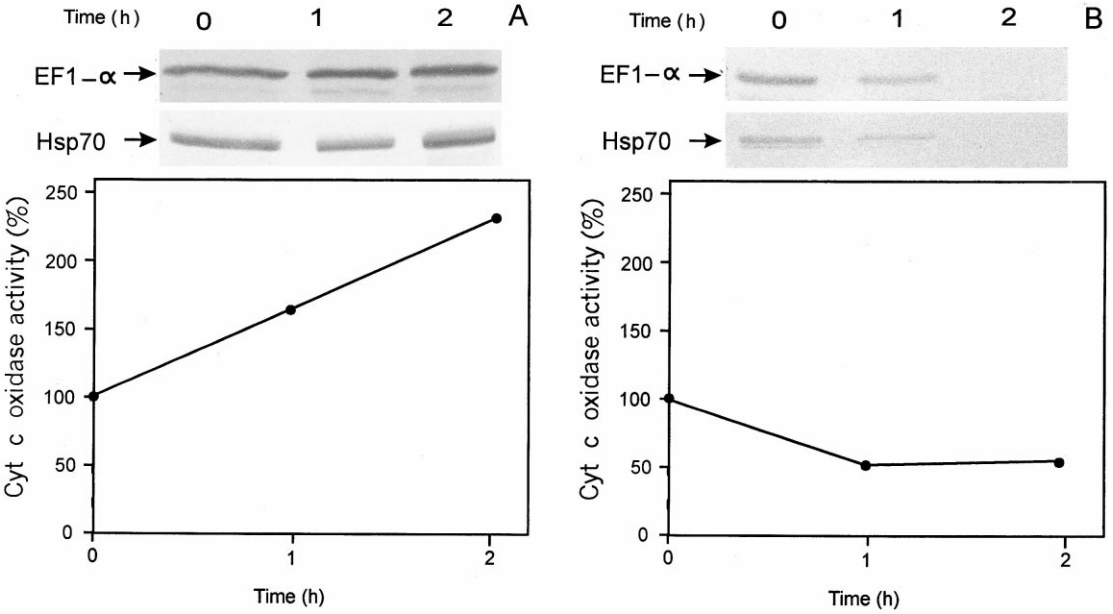

Fig. 1. Non-selective autophagy during N-starvation of glucose-grown *H. polymorpha* cells. WT *H. polymorpha* was grown on mineral media containing glucose as a carbon source and ammonium sulphate as a nitrogen source. Cells in the mid-exponential growth phase were harvested and shifted ($T=0$ h) to glucose-containing medium with (A) or without the nitrogen source (B). Samples were taken at different time points after the shift. Western blots prepared from crude extracts were decorated with antibodies against the cytosolic marker proteins EF1-α and Hsp70. Equal amounts of protein were loaded per lane. Cytochrome *c* oxidase activity was used as a mitochondrial marker. The activity is expressed as a percentage of the initial value ($T=0$ h), which was set to 100%.

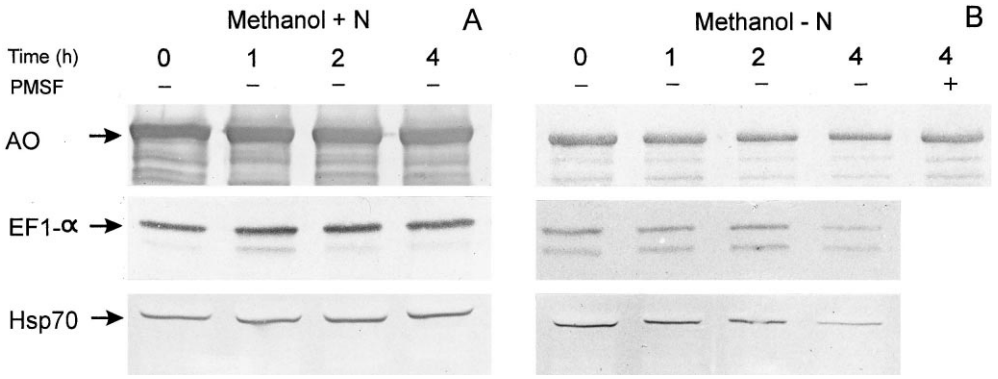

Fig. 2. Non-selective autophagy during N-starvation in methanol-grown cells. *H. polymorpha* WT cells were grown in MM containing methanol and ammonium sulphate. Cells were harvested and resuspended ($T=0$ h) in methanol-containing media with (A) or without (B) a nitrogen source in the absence (−) or presence (+) of PMSF. Samples were taken at 0, 1, 2 and 4 h after the shift. Western blots were prepared from crude extracts and decorated with antibodies against AO as peroxisomal marker or EF1-α and Hsp70 as cytosolic markers. Equal amounts of protein were loaded per lane.

using glucose (0.5% w/v) or methanol (0.5% v/v) as carbon sources. Leucine was added when required (final concentration 30 mg l^{-1}). For N-starvation conditions, cells were extensively precultivated and grown to the mid-exponential growth phase. These cells were collected by centrifugation, washed once with prewarmed MM without carbon and N source and subsequently resuspended in MM containing glucose or methanol, but lacking ammonium sulphate, leucine and yeast extract. Where indicated 1 mM phenylmethylsulphonyl fluoride (PMSF) was added from a 1 M stock solution in 100% dimethylsulphoxide.

2.2. Biochemical methods

Preparation of crude extracts [16], determination of protein concentrations [17] and sodium dodecyl sulphate–polyacrylamide gel electrophoresis [18] were performed by established procedures. Western blotting was performed using the Protoblot immunoblotting system (Promega Biotec). Alcohol oxidase (AO) [19] and cytochrome *c* oxidase [20] were assayed as described before.

2.3. Electron microscopy

Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as detailed before [16]. Immunolabeling was performed on ultrathin sections of unicryl-embedded cells, using specific antibodies against AO and gold-conjugated goat anti-rabbit antibodies (GAR-gold) according to the instructions of the manufacturer (Amersham Corp., Arlington Heights, IL, USA).

3. Results

3.1. Nitrogen-starvation induces autophagy in H. polymorpha

In mammalian cells and in the yeast *S. cerevisiae* non-selective autophagy is induced under N-starvation conditions and occurs by a mechanism defined as macroautophagy [2]. Although selective peroxisome degradation has extensively been studied in the yeast *H. polymorpha*, non-selective autophagy was never reported for this yeast. To study whether non-selective autophagy occurs in N-depleted *H. polymorpha*, cells grown on glucose in the presence of nitrogen were transferred to fresh glucose media that lacked any nitrogen source. Western blotting experiments revealed that the levels of the cytosolic marker proteins elongation factor 1-α (EF1-α) and heat shock protein 70 (Hsp70) gradually decreased after the shift to N-limitation (Fig. 1B), whereas in the presence of nitrogen the levels of these proteins slightly increased (Fig. 1A). Similarly, cytochrome *c* oxidase activity, measured as a mitochondrial marker, increased in control cultures grown in the presence of nitrogen (Fig. 1A), but decreased in cultures that were shifted to media that lacked any nitrogen source (Fig. 1B). Similar results were obtained using methanol-grown cells, except that also peroxisomal AO decreased upon the shift of cells to N-starvation conditions (Fig. 2B). In control experiments, in which cells were transferred to fresh, N-containing media, the levels of this protein slightly increased (Fig. 2A). In identical experiments performed in the presence of PMSF, an inhibitor of vacuolar proteases [21], the above-described decrease in the levels of the various proteins analysed was significantly reduced (Figs. 2B and 3). This phenomenon

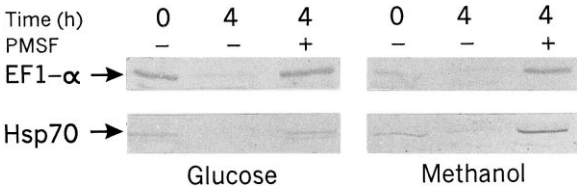

Fig. 3. The effect of PMSF on nitrogen-starvation-induced autophagy. *H. polymorpha* WT cells growing in the presence of nitrogen on glucose or methanol were shifted ($T=0$ h) to media lacking nitrogen in the absence (−) or presence (+) of 1 mM PMSF. Samples were taken at $T=0$ h and 4 h. Western blots were prepared from crude extracts and decorated with antibodies against the cytosolic marker proteins EF1-α and Hsp70. Equal amounts of protein were loaded per lane.

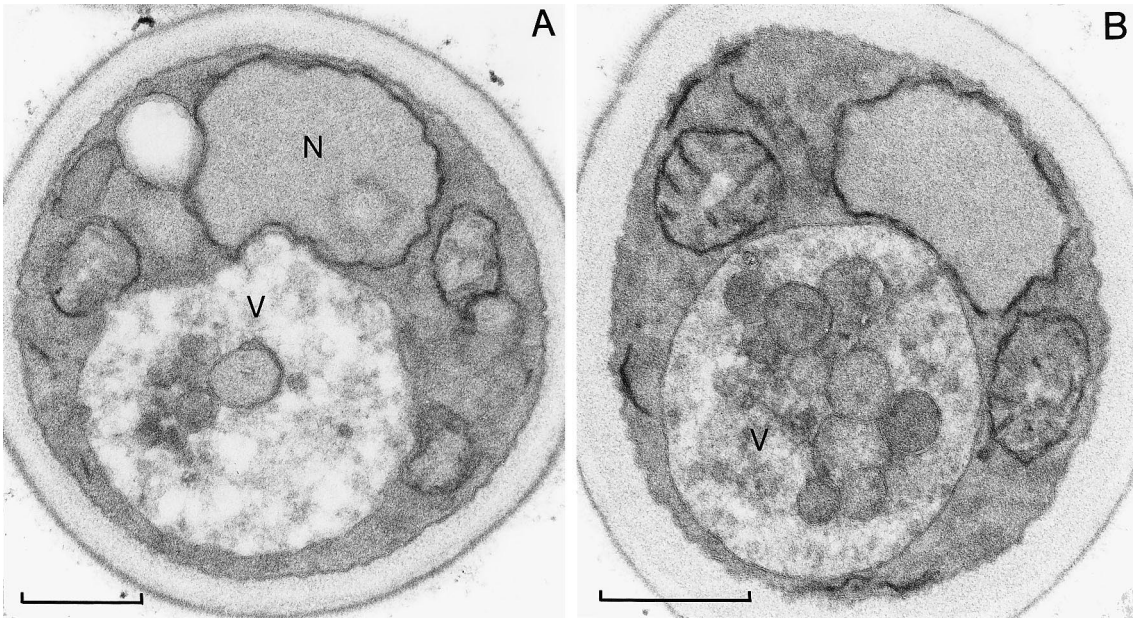

Fig. 4. PMSF causes accumulation of autophagic bodies in the vacuole. *H. polymorpha* WT cells, grown on glucose in the presence of N, were shifted to N-lacking glucose media and incubated for 4 h. The vacuole of the N-depleted cells contains cytoplasmic material (autophagic bodies, A), which accumulated in the vacuole when the incubation was performed in the presence of 1 mM PMSF (B). The electron micrographs are of $KMnO_4$-fixed cells. N = nucleus; V = vacuole. Bar = 0.5 μm.

was accompanied by the accumulation of cytoplasmic components (Fig. 4A,B; so-called 'autophagic bodies') in the central vacuole indicating that the observed reduction in protein decrease in the presence of PMSF was indeed due to a retarded degradation.

3.2. During nitrogen-starvation organelles and cytosol are degraded by a mechanism that resembles microautophagy

Upon a shift of *H. polymorpha* cells grown on methanol in the presence of nitrogen (Fig. 5A) to N-depletion conditions, protrusions of the vacuolar membrane appeared in the vicinity of peroxisomes (Fig. 5B, arrows). Subsequently, the organelles became completely boxed in by vacuolar profiles (Fig. 5C). At later stages the cells were characterised by the presence of large vacuoles, whereas the bulk of the peroxisomes that initially occurred in these cells had disappeared (Fig. 5D). These observations suggest that protrusions from the central vacuole surround single peroxisomes during N-starvation-induced autophagy.

To analyse this process in more detail serial sections were prepared from methanol-grown cells that were shifted for 2 h to N-starvation conditions. In Fig. 6 a typical example is shown in which one out of a cluster of peroxisomes is surrounded by vacuolar profiles. Following one of these profiles in subsequent sections demonstrates that the peroxisome is enclosed by longer, interconnected tubular vacuolar structures. In contrast, mitochondria and portions of the cytosol are generally taken up through invaginations of the vacuolar membrane (Fig. 7). A similar mechanism is operative for the degradation of cell components in N-starved glucose-grown cells (data not shown).

3.3. Analysis of pdd1-201 and pdd2-4 mutants during nitrogen starvation

The *H. polymorpha* mutants *pdd1-201* and *pdd2-4* are defective in selective peroxisome degradation [14]. Mutant *pdd1-201* is impaired in an initial stage of the degradation process, namely the sequestration of peroxisomes by membranes. In *pdd2-4* cells, fusion of the vacuole with sequestered peroxisomes is blocked. To test whether these mutants are also impaired in N-starvation-induced peroxisome degradation, methanol-grown cells of both strains were shifted to fresh methanol media lacking a nitrogen source. As shown in Fig. 8, AO activity gradually decreased in WT and *pdd2-4* cells upon the transfer of cells to N-limitation. In contrast, in *pdd1-201* cells AO activity slightly increased under the same conditions. These findings indicate that in mutant *pdd1-201* N-starvation-induced autophagy is blocked, whereas this process still normally functions in mutant *pdd2-4*.

Electron microscopy revealed that N-starved *pdd1-201* cells were characterised by the presence of several peroxisomes, which were not engulfed or taken up by the vacuole (Fig. 9A). Immunocytochemistry confirmed that AO protein was confined to the matrix of the peroxisomes and invariably absent in vacuoles, indicating that vacuolar degradation of peroxisomes did not occur. In *pdd2-4* cells, however, AO protein was detected both in the peroxisomal matrix and in the vacuole following N-limitation, indicat-

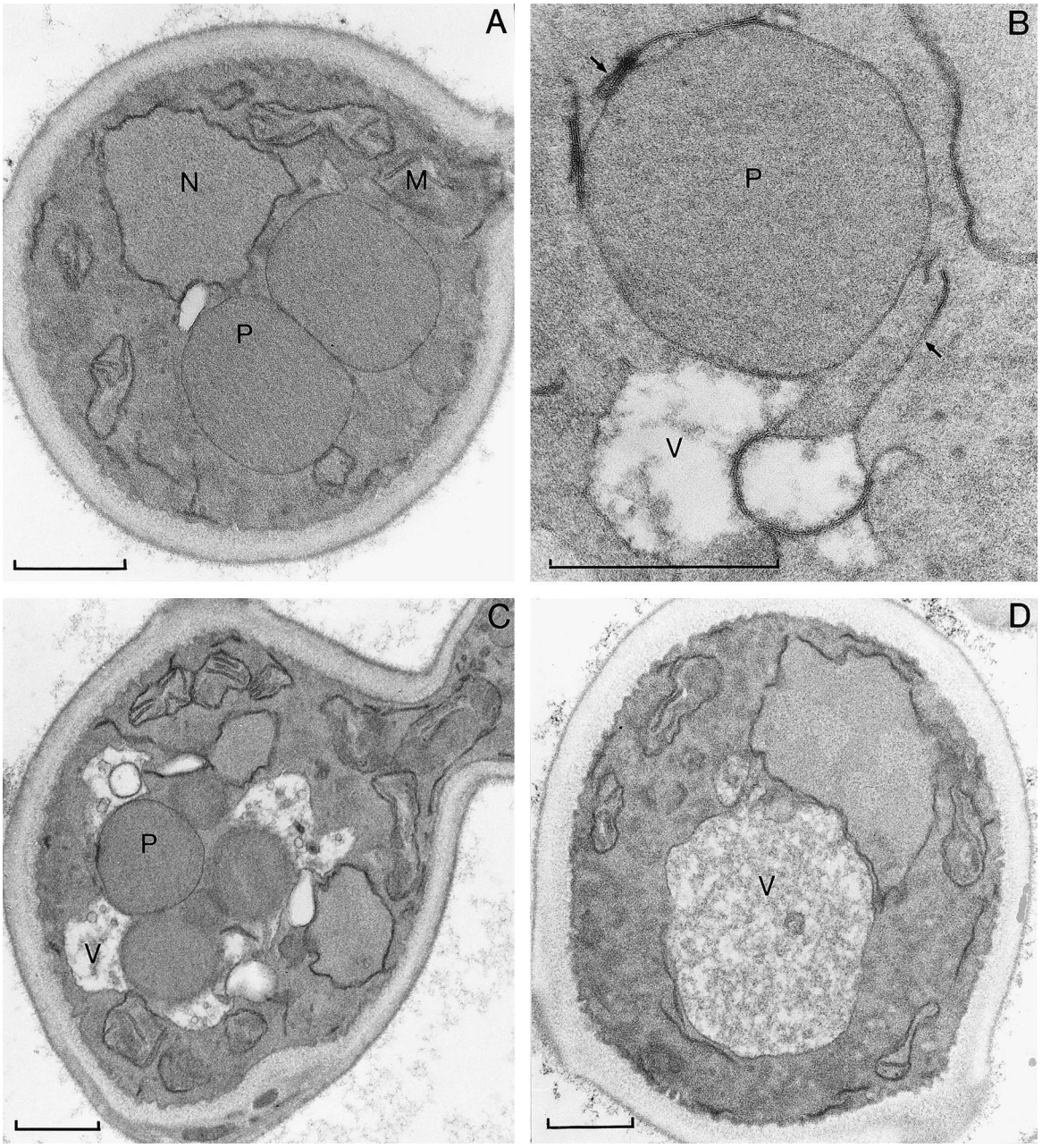

Fig. 5. Different stages of peroxisome degradation during N-starvation. *H. polymorpha* WT cells growing exponentially on methanol in the presence of nitrogen are characterised by several large peroxisomes (A). Two hours after the shift to methanol media lacking nitrogen peroxisomes are sequestered by the vacuole (B, C). Note the vacuolar membranes close to the peroxisome (B, arrows). Four hours after the shift, the central vacuole had increased in size, whereas the number of peroxisomes was reduced (D). $KMnO_4$-fixation. M = mitochondrion; N = nucleus; P = peroxisome; V = vacuole. Bar = 0.5 μm.

ing that in *pdd2-4* cells non-selective degradation of peroxisomes normally takes place (Fig. 9B).

4. Discussion

4.1. Induction of non-selective autophagy during N-starvation

In this report we show that cytosolic and organellar proteins are degraded in *H. polymorpha* cells that are placed under N-starvation conditions. In the presence of PMSF, an inhibitor of vacuolar proteases, the degradation of these proteins was retarded, resulting in the accumulation of cytoplasmic components in the vacuolar lumen. These findings indicate that, as in various other organisms (e.g. bakers yeast and mammals [2]), in N-limited *H. polymorpha* cells cytoplasmic material is taken up by the vacuole where it is subsequently digested, a process called non-selective autophagy.

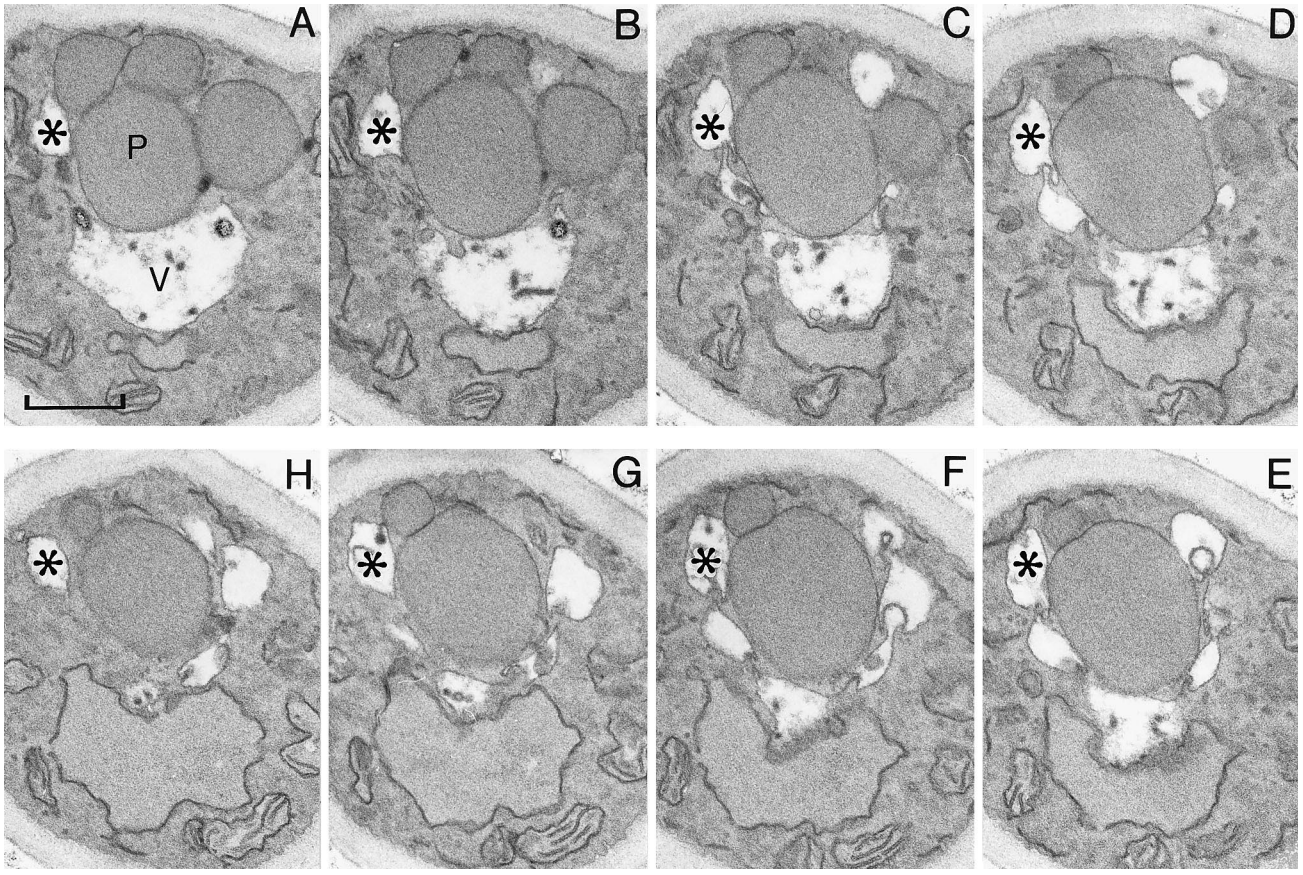

Fig. 6. Serial sections of methanol-grown N-limited WT cell showing the initial stage of the uptake of a peroxisome. The cell contains a cluster of peroxisomes (A). In subsequent sections (B–E) one peroxisome is surrounded by vacuolar profiles. Following one of these profiles (asterisk) reveals that this represents a tubular structure that is connected with other vacuolar profiles and the central vacuole. $KMnO_4$-fixation. P = peroxisome; V = vacuole. Bar = 0.5 μm.

4.2. *In H. polymorpha non-selective autophagy occurs via microautophagy*

By detailed morphological studies using serial sections, we demonstrated that in N-limited *H. polymorpha* cells cytoplasmic components are taken up by the vacuole without prior sequestration by membranes. This process therefore differs from non-selective autophagy described for *S. cerevisiae*, in which the cytoplasmic components are sequestered prior to uptake in the vacuole. Non-selective

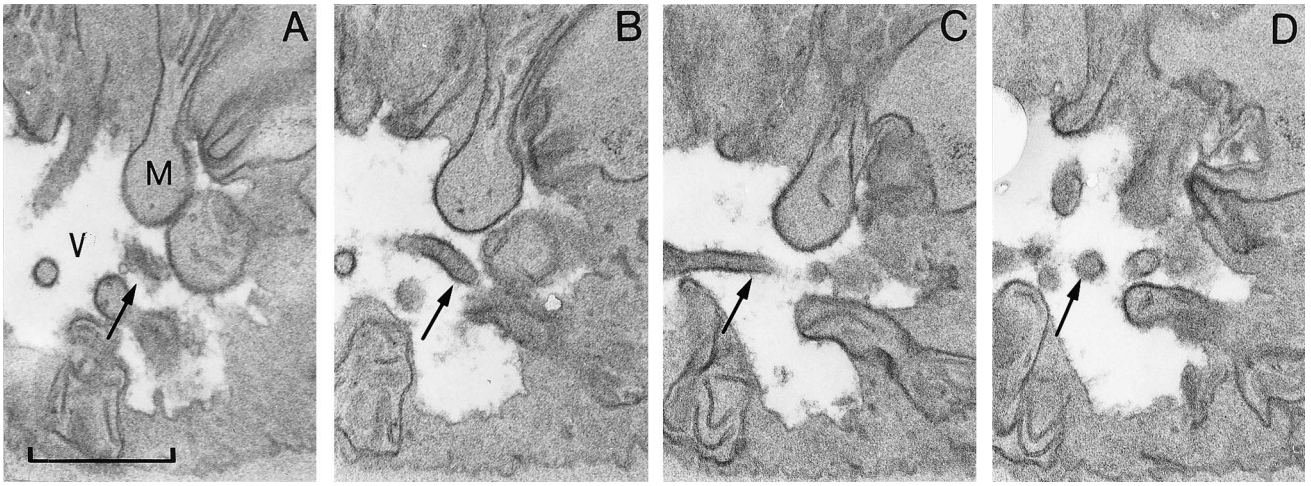

Fig. 7. Serial sections of a methanol-grown N-limited WT cell showing the uptake of cytosol and mitochondria in the central vacuole. Cytosol and mitochondria are taken up via invaginations of the vacuole. The cytosol appears as finger-like protrusions (arrow) in the vacuole. $KMnO_4$-fixation. V = vacuole; M = mitochondrion. Bar = 0.5 μm.

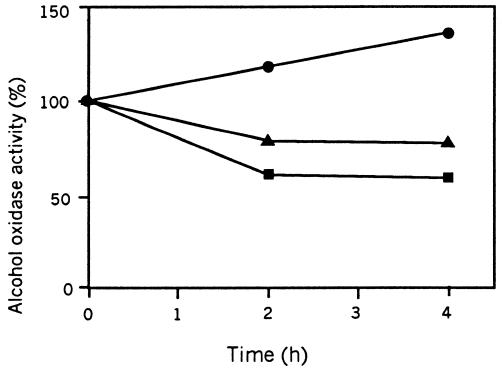

Fig. 8. Non-selective peroxisome degradation in *H. polymorpha pdd* mutants. WT *H. polymorpha* and mutants *pdd1-201* and *pdd2-4* were grown on methanol in the presence of a nitrogen source. At the mid-exponential growth phase the cultures were harvested and resuspended ($T=0$ h) in methanol media lacking nitrogen. The enzyme activity of AO (peroxisomal marker) was measured at different time points after the shift. Activities are expressed as a percentage of the value at $T=0$ h, which was set to 100%. ■ – WT, ● – *pdd1-201*, ▲ – *pdd2-4*.

autophagy in *H. polymorpha* resembles microautophagy as it involves the invagination of the vacuolar membrane (cytosol, mitochondria) or the formation of protrusions from this organelle (peroxisomes). This remarkable difference in the uptake mechanism of peroxisomes compared to other cell constituents may very well be related to the fact that peroxisomes in methanol-grown *H. polymorpha* are relatively large and rigid, since they contain a crystalline matrix that consists of AO molecules [22]. At the molecular level, however, these processes are presumably very similar because in both cases homotypic fusion of vacuolar membranes is the major event for incorporation of the components to be degraded. The formation of autophagosomes (i.e. sequestration of parts of the cytoplasm by membrane structures) as observed in N-limited *S. cerevisiae* [23] was never observed in N-limited *H. polymorpha* cells. Apparently, in this yeast N-starvation induces turnover of the cytoplasm by macroautophagy. Also in N-limited mammalian cells non-selective autophagy occurs by macroautophagy. These findings indicate that N-starvation apparently can trigger different mechanisms of autophagy, which may vary with the organism studied.

4.3. Mutant pdd1-201 is blocked in selective and non-selective peroxisome degradation

The *H. polymorpha pdd1-201* mutant is defective in an early stage of glucose- or ethanol-induced peroxisome degradation and unable to sequester individual peroxisomes by membranes [14]. We show here that *pdd1-201* is also impaired in non-selective autophagy induced by N-limitation. These data reveal that although both processes involve mechanistically distinct processes (macro- versus microautophagy), they share common genes. *H. polymorpha PDD1* encodes a protein that is most likely the functional homologue of *S. cerevisiae* Vps34p [24]. *S. cerevisiae vps34* mutants are defective in several processes that are related to the vacuole (osmoregulation, vacuolar segregation, sorting of vacuolar proteases and endocytosis) [25,26].

S. cerevisiae Vps34p is bound to membranes via the integral membrane protein Vps15p, a protein essential for activation of Vps34p. Recently, we showed that *P. pastoris* Vps15p is also necessary for glucose- and ethanol-induced peroxisome degradation, suggesting that a

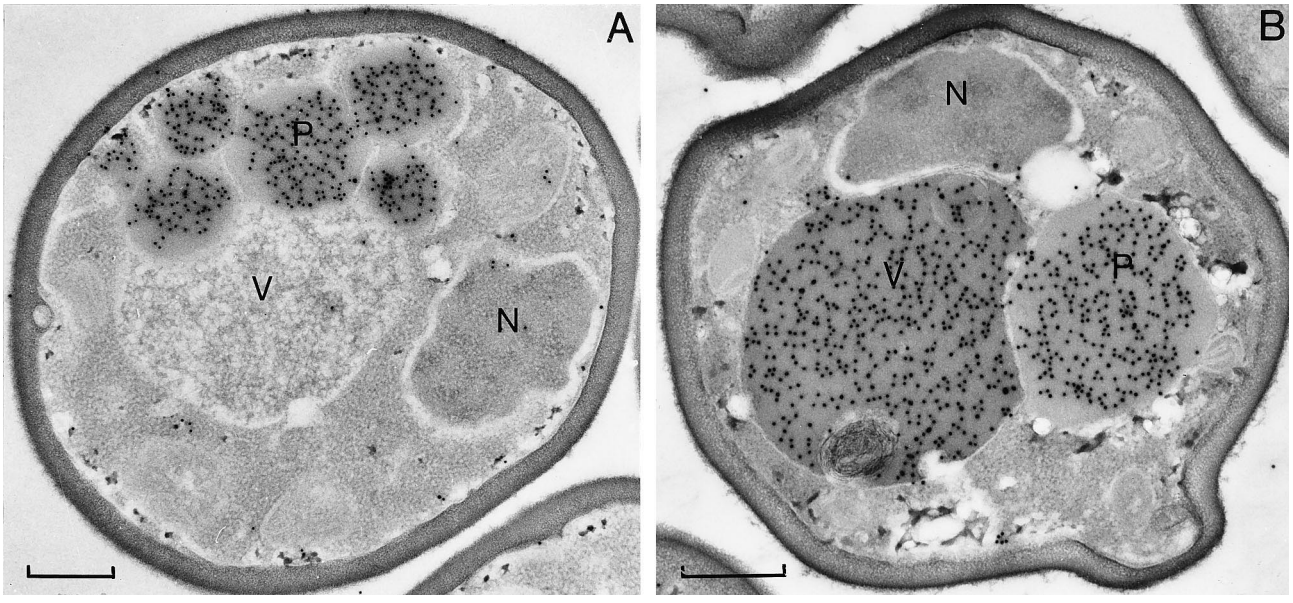

Fig. 9. Morphology of methanol-grown *pdd1-201* and *pdd2-4* cells subjected to N-starvation. Ultrathin sections of glutaraldehyde-fixed cells were labeled with antibodies against AO. (A) Four hours after the shift of *pdd1-201* cells to N-starvation conditions peroxisomes are still abundant in the cytosol. AO protein is confined to the peroxisomes and not observed in the vacuole, indicating that degradation has not occurred. (B) In *pdd2-4* cells shifted for 4 h to N-limitation few peroxisomes are present in the cytosol, whereas the vacuole contains AO protein, indicating uptake and degradation of peroxisomes in the vacuole. P = peroxisome; V = vacuole; N = nucleus. Bar = 0.5 μm.

complex similar to *S. cerevisiae* Vps34p–Vps15p functions in peroxisome turnover in methylotrophic yeasts [27]. Vps34p phosphorylates phosphatidylinositol molecules (PtdIns) at the 3 position of the inositol ring. Evidence has been presented that phosphorylation of PtdIns in membranes is important to recruit effector proteins to specific locations and/or to influence their activity. These effector proteins are characterised by a Zn^{2+} binding ring finger (designated FYVE domain) that is responsible for binding to phosphorylated PtdIns molecules in the membrane. In *S. cerevisiae* different FYVE domain proteins have been identified. Three of them, Vac1p, Vps27 and Fab1, have been further characterised [28,29]. These studies suggested that PtdIns3-kinase may modulate multiple effector proteins (FYVE proteins) involved in different pathways of membrane trafficking to and from the vacuole. In line with this hypothesis *H. polymorpha* Pdd1p may function in membrane trafficking or fusion processes related to degradation of peroxisomes by the vacuole. As *H. polymorpha pdd1-201* and *S. cerevisiae vps34* and *vps15* are defective in vacuolar protein sorting, these cells have significantly reduced vacuolar protease activities. The defects in peroxisome degradation in *H. polymorpha pdd1-201* are, however, not due to the absence of protease activity, because in *pdd1-201* cells peroxisomes do never enter the vacuole, neither upon a shift to glucose- or ethanol-containing media [14] nor during N-starvation (this paper). Hence, the *pdd1-201* mutation causes a block at an early stage in both microautophagy and macroautophagy that occurs prior to uptake in the vacuole. Likely speculations are that Pdd1p is involved in tagging the organelles to be degraded or, alternately, in organellar sequestration. As documented extensively, selective peroxisome degradation in *H. polymorpha* is initiated by the sequestration of the organelle from the cytosol, forming an autophagosome. It can be envisaged that the formation of autophagosomes may involve membrane fusion events. This could be an explanation for the finding that small membranous structures appeared near peroxisomes in *pdd1-201* cells upon a shift to glucose media [24]. Possibly, these structures are unable to fuse into larger membranes that sequester the organelle in the absence of the Pdd1p function. Hence, various possibilities are open for membrane fusion events in which Pdd1p can play a role and require further investigation.

4.4. *pdd2 is specifically involved in glucose- or ethanol-induced peroxisome degradation*

The *H. polymorpha PDD2* gene is known to be involved in selective peroxisome degradation [14]. In methanol-grown *H. polymorpha pdd2-4* not the peroxisomal sequestration, but the fusion of these compartments with the vacuole is impaired. Possibly, Pdd2p is involved in heterotypic fusion of the autophagosome with the vacuole, a process that does not occur during N-starvation-induced microautophagy in *H. polymorpha*. At present, *H. polymorpha pdd2-4* is the only mutant known to be specifically involved in selective peroxisome degradation by macroautophagy. Also for other yeast species such mutants have not been described yet. We are currently cloning the corresponding gene of this intriguing mutant.

Acknowledgements

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